Propane and *n*-Butane Oxidation by *Pseudomonas putida* GPo1

Erika L. Johnson and Michael R. Hyman*

Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27695-7615

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Propane and *n*-butane inhibit methyl tertiary butyl ether oxidation by *n*-alkane-grown *Pseudomonas putida* GPo1. Here we demonstrate that these gases are oxidized by this strain and support cell growth. Both gases induced alkane hydroxylase activity and appear to be oxidized by the same enzyme system used for the oxidation of *n*-octane.

The *n*-alkane-oxidizing activity of *Pseudomonas putida* GPo1 is a model for the bacterial oxidation of *n*-alkanes other than methane (11). In this strain, *n*-alkane oxidation is initiated by alkane hydroxylase and the *n*-alkane growth substrate range of the bacterium (C₅ to C₁₃) matches the *n*-alkane substrate range of the native hydroxylase (10–12). We recently demonstrated that alkane hydroxylase in strain GPo1 oxidizes the gasoline oxygenate MTBE to tertiary butyl alcohol (TBA) (9). Propane and *n*-butane inhibited this activity, and these effects appeared to be due to a competitive interaction between hydroxylase substrates. However, no evidence for oxidation of these gases was presented, and neither gas is currently recognized as an alkane hydroxylase substrate.

In this study we first examined whether strain GPo1 oxidized gaseous *n*-alkanes (C₁ to C₄) during growth on a model *n*-alkane, *n*-octane. No consumption of either methane or ethane occurred during growth on *n*-octane, while both propane (~20%) and *n*-butane (~60%) were consumed under the same conditions (Fig. 1). In abiotic incubations, ≤20% of *n*-octane (Fig. 1) and ≤3% of gaseous *n*-alkanes were lost over the same time period (not shown). In a separate experiment, we determined the final culture densities (optical densities at 600 nm [OD₆₀₀]) and protein contents of cultures grown on C-limiting *n*-octane with gaseous *n*-alkanes. After exhaustion of *n*-octane (5 days), the average culture density and protein concentration for three separate cultures were 0.22 and 46 μg/ml, respectively, for cultures grown on *n*-octane alone. No increase in culture density was observed with methane or ethane, while the average (*n* = 3) final culture densities and protein concentrations for cultures containing propane were 0.43 and 62 μg/ml or for cultures containing *n*-butane were 1.1 and 148 μg/ml, respectively.

Propane and *n*-butane were also examined as potential independent *n*-alkane growth substrates. Cultures were established with propane, *n*-butane, *n*-pentane, or *n*-octane added individually as sole substrates. The time courses of *n*-pentane and *n*-octane consumption were almost identical, and changes in culture density closely reflected *n*-alkane consumption (Fig. 2). In contrast, no detectable *n*-alkane consumption or increases in culture density were initially observed in *n*-butane-

containing cultures. However, after ~4 days, *n*-butane consumption was initiated and accompanied by a corresponding increase in culture density. No discernible effect on this lag phase or final culture density was observed when cultures were supplemented with fourfold-higher (~0.7% [vol/vol] gas phase) concentrations of the main hydrocarbon contaminant (isobutane) present in our *n*-butane. Unlike with *n*-butane, no gas consumption or changes in culture density were observed for propane-containing cultures. However, slow growth was observed with severalfold-higher initial propane concentrations (Fig. 3), and no consistent differences were observed using either instrument-grade (99.9%) or research-grade (99.993% minimum-purity) propane. Use of correspondingly higher *n*-butane concentrations decreased the previously observed lag phase. This suggests growth on *n*-butane is strongly impacted by dissolved gas concentration and is unlikely to reflect the growth of a subset of strains with altered responses to *n*-butane. No growth over 10 days (final OD₆₀₀, ≤0.01) occurred when the OCT plasmid- and alkane hydroxylase-deficient strain GPo12 (9) was incubated with the maximum propane and *n*-butane concentrations used above.

A potential role of alkane hydroxylase in propane and *n*-butane oxidation was explored by comparing the specific alkane hydroxylase-dependent MTBE-oxidizing activities of strains GPo1 and GPo12 with the results of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis-immunoblot analysis of total cell proteins using an anti-GPo1 alkane hydroxylase (AlkB) antiserum. High specific MTBE-oxidizing activities (18 to 38 nmol min⁻¹ mg total protein⁻¹) were observed for strain GPo1 grown on propane, *n*-butane, and *n*-octane. Little or no activity (≤0.1 to 3 nmol min⁻¹ mg total protein⁻¹) was observed with strains GPo1 and GPo12 grown on alcohols or dextrose-containing medium (Fig. 4). The immunoblot analysis showed a strongly cross-reacting ~40-kDa polypeptide in strain GPo1 grown on *n*-octane, *n*-butane, or propane and but not in cells grown on alcohols or dextrose-containing medium. No cross-reaction was observed for strain GPo12, irrespective of growth substrate.

Overall, our results demonstrate that strain GPo1 not only oxidizes propane and *n*-butane during growth on *n*-octane but also utilizes these two gases as independent growth substrates. These effects all appear to be manifestations of the broad substrate ranges of the enzymes involved in *n*-alkane oxidation and the regulatory elements that control their expression. For example, the gaseous *n*-alkane consumption (Fig. 1) and in-

* Corresponding author. Mailing address: Department of Microbiology, North Carolina State University, Raleigh, NC 27695-7615. Phone: (919) 515-7814. Fax: (919) 515-7867. E-mail: michael_hyman@ncsu.edu.

FIG. 1. Time course of *n*-octane and gaseous *n*-alkane consumption by *P. putida* GPo1. Cultures of *P. putida* GPo1 were grown in sealed glass serum vials (160 ml) containing mineral salts medium (25 ml) (8). The cells were grown in batch culture on C-limiting amounts of *n*-octane (10 μ l [0.04%, vol/vol, liquid phase]) in the presence or absence of gaseous *n*-alkanes (10 ml [$\sim$ 7.5%, vol/vol, gas phase]). Concentrations of *n*-alkanes were determined by gas chromatography, as described previously (8). The figure shows the time course for *n*-octane consumption for uninoculated abiotic control cultures containing ($\diamond$) *n*-octane and inoculated cultures containing ($\blacklozenge$) *n*-octane, ($\square$) *n*-octane plus methane, (Δ) *n*-octane plus ethane, ($\circ$) *n*-octane plus propane, and (∇) *n*-octane plus *n*-butane. The figure also shows the corresponding time course for gaseous *n*-alkane consumption for cultures containing *n*-octane and ($\blacksquare$) methane, ($\bullet$) ethane, ($\blacklozenge$) propane, or ($\blacktriangledown$) *n*-butane. The data plotted are the averages and ranges from two replicate cultures.

creased biomass production observed during propane and *n*-butane cooxidation suggests that enzymes induced by *n*-octane not only initiate gaseous *n*-alkane oxidation but also further oxidize the initial oxidation products generated from these

FIG. 2. Time course of growth of *P. putida* GPo1 on *n*-butane and liquid *n*-alkanes. A series of cultures of *P. putida* GPo1 were prepared and analyzed by gas chromatography, as described for Fig. 1. The figure shows the time course of culture growth (OD_{600}) (open symbols) and *n*-alkane consumption (filled symbols) for cultures incubated with ($\bullet$, $\circ$) *n*-octane (10 μ l [0.04%, vol/vol, liquid phase]), ($\blacktriangle$, $\triangle$) *n*-pentane (10 μ l [0.04%, vol/vol, liquid phase]), ($\blacksquare$, $\square$) *n*-butane (10 ml [$\sim$ 7.5%, vol/vol, gas phase]), and ($\blacktriangledown$, $\triangledown$) propane (10 ml [$\sim$ 7.5%, vol/vol, gas phase]). The data plotted show the averages and ranges of values from two replicate cultures. d, days.

FIG. 3. Time course of growth of *P. putida* GPo1 on propane or *n*-butane. Cultures of *P. putida* GPo1 were grown on propane or *n*-butane, as described for Fig. 1. The figure shows the changes in culture density (OD_{600}) versus time for cultures grown with ($\blacklozenge$) $\sim$ 15%, ($\diamond$) $\sim$ 30%, and ($\blacktriangledown$) $\sim$ 45% (vol/vol, gas-phase *n*-butane); ($\blacksquare$) $\sim$ 15%, ($\bullet$) $\sim$ 30%, and ($\blacktriangle$) $\sim$ 45% (vol/vol, gas phase) instrument-grade ($\sim$ 99% purity) propane; and ($\square$) $\sim$ 15%, ($\circ$) $\sim$ 30%, and ($\triangle$) $\sim$ 45% (vol/vol, gas phase) research purity (99.993%) propane. The data plotted show the averages and ranges of values from three replicate cultures. d, days.

gases. The high levels of the $\sim$ 40-kDa hydroxylase component observed only in gaseous *n*-alkane-grown cells (Fig. 4) suggests that these gases, and not their corresponding primary alcohols, act as inducers of alkane-oxidizing activity.

It is unclear why the gaseous *n*-alkane-oxidizing activity of this model strain has not been previously described. *n*-Octane-grown cells are known to hydroxylate propylene and 1-butene, alkene analogs of propane and *n*-butane (4, 5), and *n*-butane is a potent inducer of GPo1-like alkane hydroxylase expression in *P. aeruginosa* strain 423 (13). In early and frequently cited

FIG. 4. MTBE-oxidizing activities and immunoblot analysis of *P. putida* GPo1 and GPo12 cells grown on various substrates. The figure shows the results of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis-immunoblot analysis of whole-cell proteins (50 μ g total protein) for *P. putida* GPo1 and GPo12 strains grown on the following substrates: (lane 1) GPo1-dextrose, (lane 2) GPo1-1-propanol, (lane 3) GPo1-propane, (lane 4) GPo1-1-butanol, (lane 5) GPo1-*n*-butane, (lane 6) GPo1-1-octanol, (lane 7) GPo1-*n*-octane, (lane 8) GPo12-dextrose, and (lane 9) GPo12-1-octanol. The figure also shows the corresponding specific MTBE-dependent TBA-generating activities of each strain grown on each substrate expressed in nmol TBA generated $\text{min}^{-1} \text{mg total protein}^{-1}$. The immunoblotting was performed using a WesternBreeze chemiluminescence immunodetection system (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturers instructions. The primary antibody, rabbit anti-AlkB (generously supplied by J. van Beilen, ETH Hönningerberg, Zürich, Switzerland), was used at a dilution of 1:2,500. The specific MTBE-degrading activity was determined using concentrated cells ($\sim$ 200 μ l, 0.6 to 3 mg total protein) added to buffer ($\sim$ 800 μ l) with MTBE (42 μ mol) in a sealed glass serum vial (10 ml). The vials were incubated at 30°C in a shaking water bath (150 rpm), and TBA production was determined by gas chromatography after 30 min, as described previously (8, 9).

descriptions of the physiology of strain GPo1, agar plates incubated with liquid *n*-alkane vapors were often used (1, 3, 6, 7), an approach that underreported the true *n*-alkane growth substrate range of this strain. Alternatively, strain GPo1 may have emerged as a model organism because of its particularly rapid growth on *n*-alkanes, such as *n*-octane. As growth on *n*-butane and propane is initiated slowly and is dependent on dissolved gas concentration, previous evaluations of propane- or *n*-butane as growth substrates either may have been conducted for an insufficient time or may have used insufficiently high gas concentrations.

This study suggests that propane is inferior to *n*-butane as a substrate. Propane was consumed threefold more slowly than *n*-butane during growth on *n*-octane (Fig. 1) despite both gases being present at equal initial concentrations and propane having a higher saturated aqueous solubility than *n*-butane (14). We have also shown that *n*-butane ($K_i = 13 \mu\text{M}$) is a more potent inhibitor of MTBE oxidation than propane ($K_i = 66 \mu\text{M}$) by strain GPo1, and it should be noted that K_i values for competitive substrates are the equivalent of the K_s values for these compounds (2). In vivo and in vitro rates of alkane hydroxylase-dependent *n*-alkane consumption are also known to steadily decrease from *n*-nonane to *n*-pentane (10). Although this might reflect progressive increases in *n*-alkane solubility and toxicity, our current results suggest that decreases in the *n*-alkane carbon chain length lead to increased K_s values and decreased V_{max} values for *n*-alkanes. Our present results suggest that these trends continue through *n*-butane but that ultimately these opposing trends conspire to effectively terminate the short-chain *n*-alkane substrate range of GPo1 alkane hydroxylase at the level of propane.

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